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High nuclease activity shown by sorghum tissue *in vitro*

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ABSTRACT

Lack of suitable genetic transformation protocol is the major bottleneck for application of transgenic approach for sorghum improvement. Available evidence from the literature and our work shows that tissue culture methodology for plant regeneration is not a limitation for the development of transgenics in sorghum. However, the block lies in the transformation process itself. In this paper we report the presence of very high levels of extra cellular and intra cellular nuclease activities which may be responsible for the poor transformation competence of the sorghum tissue. Further we report treatments that can mitigate the nuclease activity without affecting the tissue culture competence of the tissue.

Key Words: Sorghum bicolor, Nuclease, Callus, Explant, EDTA, Inhibition

1. INTRODUCTION

Availability of an efficient regeneration system and the protocol for high frequency genetic transformation is a prerequisite for development of transgenics. Sorghum the fifth most important cereal crop of the world has been found to respond positively for regeneration of plants *in vitro* from callus. High frequency callusing and regeneration of plants have been established in many varieties of sorghum using different explants and cultural parameters (Bhaskaran et al, 1987; Bhat and Kuruvinashetty, 1995; Gendy et al, 1996; George and Eapea 1988; Guo and Liang, 1993; KaeppJer and Pedersen, 1997; Kostina et al, 1996; Nahdi et al, 1995; Nakamura et al, 1997;; Rao and Kishore, 1989; 1995 Wei and Xu, 1990). In spite of several efficient tissue culture protocols, in very few cases genetic transformation of sorghum has been reported (Able et al, 1998;; Battraw and Hall 1991; Godwin et al, 1993; Casas et al, 1993; 1997; Hagio et al, 1991; Zhu et al, 1998;). To date *Agrobacterium* mediated genetic transformation of sorghum has not been found to be easy. Further, direct gene transfer via protoplast and electroporation has not been successful. Therefore, particle bombardment method is the method of choice for sorghum because it is independent of explant or genotype. Few reports have appeared on the successful application of this technique to transform sorghum (Zhu et al, 1998; Able et al, 1998). But the frequency of transformation is very much low compared to other cereal crops. This is a surprising observation in sorghum because even in plants with low frequency of regeneration such as legumes there is considerable success in transforming them.

The presence of nuclease activity was found to be one of the reasons for the poor transformation competency in some of the plants which were very-difficult to transform (Vischi and Marchetti, 1997; Eady et al, Jardinaud et al, 1993). So it

was hypothesised that one of the problems in genetic transformation of sorghum by particle bombardment would be the presence of extensive nuclease activity in the explants and experiments were conducted to test this aspect In this communication we report the presence of extensive nuclease activity in sorghum tissue in vitro and a treatment method to mitigate this problem.

2. MATERIAL AND METHODS

2.1. Plant Material

All tissues tested (immature embryo, immature inflorescence, immature inflorescence derived callus, shoot apex, shoot tip derived callus and mature embryo) were obtained from the sorghum cultivar M35-1. Immature embryo and immature inflorescence were collected from plants grown in the field. For other experiments *in vitro* grown plants were used. Callus was induced from immature embryo, mature embryo, immature inflorescence, shoot apex and shoot tips in MS media supplemented with different concentrations and combinations of 2AD and kinetin.

2.2. Nuclease activity tests

NucJease activity was assayed in immature embryo, mature embryo, immature inflorescence, shoot apex, shoot tips and callus generated from fliese explants. Twenty mg of each tissue was suspended in I20µl of MS medium (Murashige and Skoog, 1962) containing 20 µl plasmid DNA (1µl/µl) and incubated for 10 min at 23° C. Five microlitres of the reaction mixture was diluted in 15 µl sterile water and submitted to electrophoresis (70 V, 2h) in a 0.7 % (W/V) agarose gel DNA degradation was determined by ethidium bromide staining and examination of the gel under UV illumination. For testing the endogenous activity, 20 mg of tissue was ground in 120 µl of MS medium and was centrifuged at 10000 x g, at 4° C for 10 minutes to remove the debris. Twenty µl of plasmid DNA (1 µl / µl) was added to the supernatant and incubated for different time intervals. The degradation profile was analysed in agarose gel as described above.

2.3. Nuclease Inhibition Tests

The efficacy of different chemicals to mitigate the nuclease activity was tested first by addition of the chemical (100 mM EDTA, 0.1 M spermidine) in the media in which the tissue was soaked or ground and the DNA degradation was checked. Subsequently, based on efficacy of the chemicals used, EDTA solution was selected It was used to treat the tissue for different time intervals and the effect of treatment on the nuclease activity was assayed by the DNA degradation assay as mentioned above. Further the treated tissue (immature inflorescence, shoot tips) were checked for growth in callusing media The ability of callus (derived from immature inflorescence and shoot tips) to regenerate after treatment was checked in regeneration media (MS media with 2mg/l BAP and 0.2 mg/1 NAA).

2.4. EDTA Treatments

Tissues were immersed in MS medium supplemented with different concentrations of EDTA (10 mM - 500 mM) for different time intervals (10 min - 8 h). *The* exogenous and endogenous nuclease activities of the treated tissue were checked as described above. The longevity of the EDTA effect was checked by soaking the tissue in MS media supplemented with 100 mM EDTA, and incubating the treated tissue in callusing medium. The incubated tissue was removed at different time intervals and the nuclease activity was tested by DNA degradation assay. One experiment was also conducted in which 100 mM EDTA was amended to callusing agar media and the tissue was incubated on this plate for different time intervals to check for inhibition of nuclease activity. Treated calli were tested for growth in callusing media and for regeneration in regeneration media.

3. RESULTS

3.1. Nuclease Activity in sorghum tissue

Very high nuclease activity was detected in calli derived from shoot tips, immature embryo, mature embryo, immature inflorescence and shoot apex when soaked in the media. All these tissues also showed equal activities when the tissue was ground and tested for DNA degradation. Within 10 min at room temperature these extracts were able to degrade large quantity of plasmid DNA completely (Fig-1). Organised tissue showed less activity when compared to calli. But organised tissue when cut into pieces and incubated on osmotic medium (used for particle bombardment experiments) for 4 hours showed increased nuclease activity. But calli in general showed very high endogenous and exogenous nuclease activity that was sufficient to degrade the plasmid DNA (all forms) in a short time. Shoot tips when bombarded with DNA coated gold or tungsten particles also showed increased nuclease activity.

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3.2. Nuclease inhibition experiments

To prevent degradation of exogenous DNA by imcleases, two types of experiments were conducted. When the nuclease inhibitors (EDTA, spermidine) were added to the MS liquid medium in which the tissue was soaked or in which the tissue was ground to make an extract. Among the inhibitors tested EDTA was found to be more effective in combating the nuclease activity (Fig- 2; Table - 1). At 100 mM concentration, EDTA completely eliminated the nuclease activity.

To test whether the successful nuclease inhibiting agent, EDTA can give protection when infiltrated into the tissue, tissue were soaked in different EDTA solutions (10-500 mM) for different time intervals (10 min- 8 h) and the tissue were tested for nuclease activity. Both endogenous and exogenous nuclease activities are prevented by the EDTA infiltration of tissue. The protection level varied with EDTA concentration and time of incubation. At 100 mM concentration of EDTA, complete protection was observed (Fig-3). When 100 mM EDTA treatment was given for different time intervals full protection is achieved by 30 minutes treatment. Beyond 30 min the treatment is not necessary. When the calli were inoculated directly onto 100 mM EDTA amended callusing or regeneration media no protection was observed.

3.3. Longevity of protection by EDTA

To test how long the infiltrated EDTA can protect the exogenous DNA from degradation, the EDTA treated tissue (100 mM for 30 min) were inoculated onto callusing media and incubated in the culture room in darkness. Aliquots of tissue were removed at different time intervals and tested for nuclease activity. The treatment was found to have protective effect on nudease activity beyond 8 rtrs (Fig-4).

Table 1

Introduction of nuclease activity by EDTA infiltration

EDTA (mM)	Duration of Infiltration								
	10 min	20 min	30 min	1 hr	2 hr	4 hr	8 hr	12 hr	24 hr
10	-	-	+	+	++	++	+++	++++	++++
25	+	+	+	++	+++	+++	++++	++++	++++
50	+	++	++++	++++	++++	++++	++++	++++	++++
75	+	++	++++	++++	++++	++++	++++	++++	++++
100	+	+++	++++	++++	++++	++++	++++	++++	++++
200	+	+++	++++	++++	++++	++++	++++	++++	++++
500	+	+++	++++	++++	++++	++++	++++	++++	++++

- No inhibition + 25 % inhibition ++50% inhibition +++ 75 % inhibition ++ 100 % inhibition

Table 2

Effect of EDTA infiltration on callusing from immature inflorescence and regeneration from immature inflorescence derived calli

S.No	Category	No. of explants cultured	Number of explants responded for callusing	No. of explants responded for regeneration	Callusing frequency (%)	Regeneration frequency (%)
1	Control	3000	2916	-	97.2 $\pm$ 2.5	-
2	EDTA treated	3000	2854	-	95.13 $\pm$ 2.1	-
3	Control	2916	-	2872	-	98 $\pm$ 1.65
4	EDTA treated	2854	-	2689	-	94 $\pm$1.89

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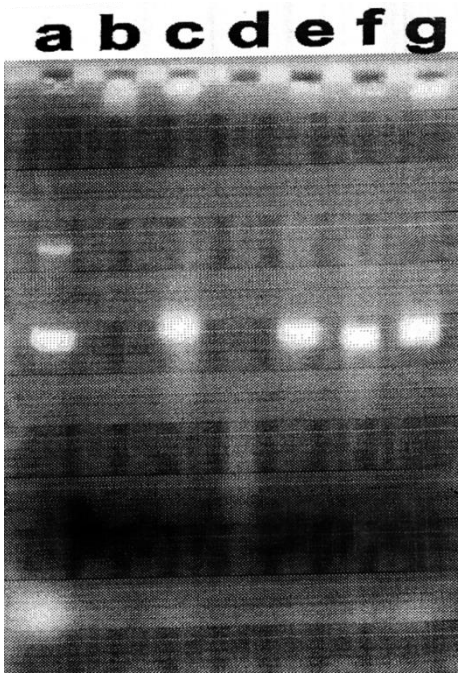

Figure 1 Nuclease activity shown by sorghum explants

a. Untreated plasmid DNA
b. Plasmid treated with extra-cellular extract of immature inflorescence derived call!
c. Plasmid treated with extra-cellular extract (containing 100 mM EDTA) extract of immature inflorescence derived calli
d. Plasmid treated with endogenous extract of immature inflorescence derived calli
e. Plasmid treated with endogenous extract (containing 100 mM EDTA) of immature inflorescence derived calli
f. Plasmid treated with extracellular extract of immature inflorescence
g. Plasmid treated with extracellular extract of shoot tips

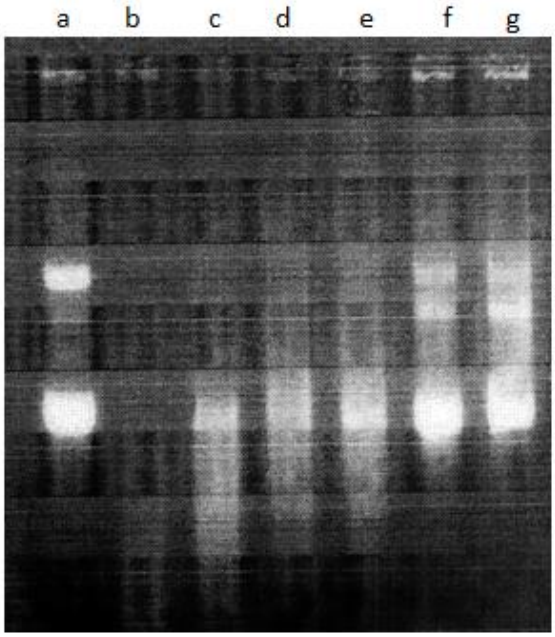

Figure 2 Effect of Different Concentration of EDTA on nuclease activity by immature inflorescence derived calli

a. Untreated plasmid DNA;
b. G-Plasmid treated with extracellular extract of EDTA infiltrated immature inflorescence derived calli

(B-10 mM, C-25 mM, D-50 mM, E-75 mM, F-100 mM, G-200 mM)

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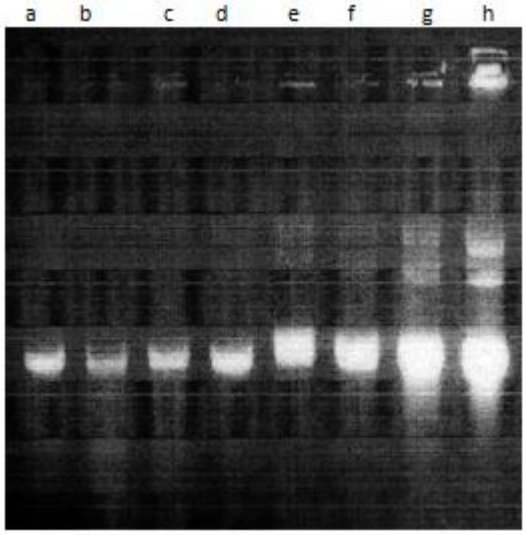

Figure 3 Effect of duration of EDTA infiltration (100 mM) on nuclease activity (the extracellular extract was made immediately after EDTA infiltration and washing and the plasmid were treated with the extract as mentioned in the Materials and methods). Slots A-G represents plasmid treated with extracellular extract made after EDTA infiltration for 30 min, 1 hrs, 2hrs, 4hrs, 8 hrs, 10 hrs, 12 hrs and 16 hrs, respectively.

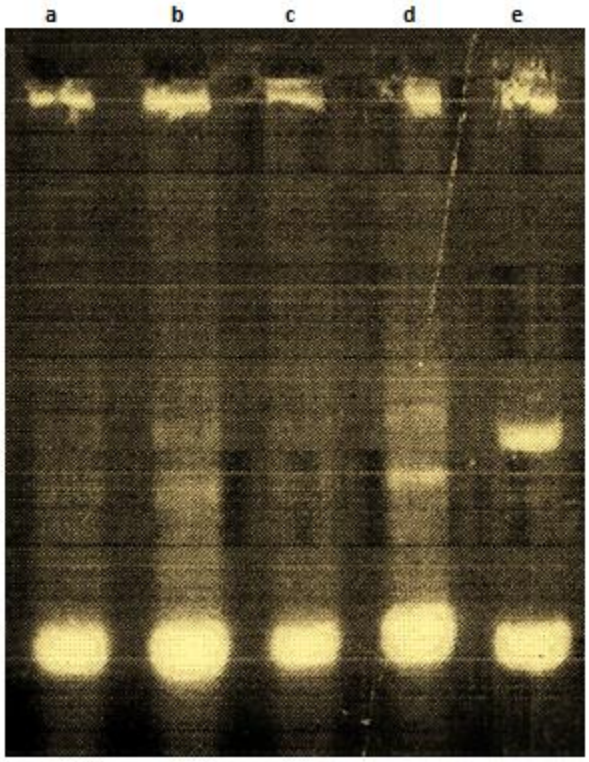

Figure 4 Effect of EDTA infiltration (100 mM, 30 min) on the extracellular nuclease activity shown by the immature inflorescence derived calli after transfer to MS basal medium (Calli were treated with 100 mM EDTA for 30 min and after through washing were inoculated onto MS based media plates. These calli were removed at different intervals and the extracellular extract was prepared as described in Materials and Methods). Slots A - E represent Plasmid treated with extracellular extract made after 2 his, 4hrs, 6hrs, 8hrs and 12hrs after EDTA treatment, respectively.

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3.4. Suitability of EDTA treated tissue for tissue culture

Experiments were conducted to test the receptive nature of the EDTA treated (nuclease silenced) tissue for tissue culture protocols (callusing and regeneration). When treated with 100 mM EDTA for 30 min and washed 3-5 times in sterile water to remove the external EDTA, shoot tips and immature inflorescence gave rise to normal callusing without significant reduction in callusing frequency, Nuclease silenced calli (derived from immature inflorescence) when inoculated onto fresh callusing media showed a initial lag of 4-5 days but started to grow normally after 5 days. When nuclease silenced calli were inoculated onto regeneration media, there was a slight reduction in regeneration frequency observed. However, they gave rise to normal shoots comparable to the untreated ones.

4. DISCUSSION AND CONCLUSION

Various reports and our results show that it is not difficult to get embryogenic caili or regenerate plants from explants like shoot tips, shoot meristem, mature embryo, immature embryo and immature inflorescence.

In spite of the availability of several efficient regeneration protocols die genetic transformation of sorghum is still not routine and challenging. This is due to the poor frequency of transformation. Various factors may contribute to this. These include, unsuitability of the gene or promoter used, lack of integration of the exogenous DNA, and degradations of the exogenous DNA before it could integrate with the host genome. Though successful transformation has been reported, there is no systematic study available with respect to comparative quantitative expression of the genes driven by different promoters in sorghum.

The second possibility of foreign DNA not integrating into the tissue and then establish itself because of die presence of many endogenous and extracellular nucleases in die plant tissue. Presence of nuclease activity in plant tissue has been reported (Ashraf et al, 1993; Broglia and Corona, 1995; Jardinaud et al, 1993; Matousek and Tupy, 1985; Mitder and Lam, 1995; 1997; Neguruiu et al, 1986, Rockel et al, 1998; Van Wot and Saunders, 1992). In most cases, the activity is induced by specific conditions. Nuclease activity is induced during the programmed cell death induced by invading pathogens. Mittler and Lam (1995; 1997) mechanical injury was also found to induce the nuclease activity in plant tissue (Mitder and Lam, 1997). These nuclease activities differ from those which get induced during senescence (Mittler and Lam, 1997) Nucleases which degrade die exogenous foreign DNA has been reported since long. Nuclease activities were found to inhibit expression of foreign DNA in cells and tissue (Ashraf et. al 1993; Broglia and Comoma 1995; Jardinaud etal 1993; Negrutiuctal, 1986, Roeckel etal 1998). Sorghum tissue examined in the present study (shoot apex, shoot tips, immature embryo, mature embryo, immature inflorescence, calli derived from these explants) showed varying levels of nuclease activity. Exogenous activity was very high in case of all the calli, irrespective of die explant from which it was derived, while organised tissue showed almost no activity which may be due to the injury caused due to cutting. All me calli showed high levels of endogenous activity also. The organised tissue showed *very low* levels of activity when tested immediately after their dissection from the plant. But they showed considerable activity after incubation for 4 h in osmotic medium used in genetic transformation experiments. But this activity is low compared to the activity showed by calli. The shoot tips when bombarded with gold or tungsten particles (coated with DNA) showed enhanced nuclease activity which may be due to the mechanical injury caused by the particles.

Several methods have been employed to prevent degradation of exogenous DNA by nucleases which include treatment with spermidine (Galston etal 1979), heparin (Case 1982) ATA (Ramon et. al 1986) NaCl (Perbal 1988) and EDTA & EGTA (Vischi and Marchetti 1997).

In the present study EDTA (which is a metal chelating agent), *when* infiltrated, could completely inhibit the nuclease activity exhibited by the sorghum tissue. A desired concentration and time is required for the inhibitor to act on the nuclease. The best treatment was found to be infiltration of 100 mM EDTA (in MS medium) for 30 min (without vacuum). The inhibitor can persist in the tissue upto 8 to 12 hrs. Which is an added advantage because after bombardment of the DNA into the tissue it will take time to integrate and establish so there will not be any degradation of this exogenous DNA at least upto 12 hrs. As the tissue infiltrated with EDTA is not only used for transformation but subsequently me tissue should give rise to callus and should be suitable for plant regeneration. Results show that though there is an initial lag in the formation or growth of callus there was not much reduction in callus induction frequency or the callus growth, EDTA infiltration reduced the regeneration frequency by 5 - 10 % but there was no morphological differences observed. These results show that EDTA infiltrated calli respond positively for tissue culture protocols which is a prerequisite for using them as explants in the particle bombardment experiments.

Transient expression of the GUS gene is widely used to establish and optimise conditions in plant transformation (Jefferson et al., 1987). When EDTA infiltrated (nuclease silenced) calli and control calli were bombarded with GUS gene constructs, there is a substantial enhancement of the number of explants showing GUS activity (data not shown). Our study demonstrates that sorghum

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tissue show extensive nuclease activity (both endogenous and exogenous) which may play an important role in determining the poor transformation frequency found in sorghum Treatment of explants with EDTA resulted in elimination of the nuclease activity which was persisting beyond 12 hours. When applied this may help in enhancing the transformation frequency because of the prevention of degradation of the introduced plasmid. The observations obtained in the present study revealing the nuclease activity in different tissue may pave a way to delineate the bottleneck in developing transgenics in sorghum.

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Conflicts of interests

The authors declare that there are no conflicts of interests.

Data and materials availability

All data associated with this study are present in the paper.

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